The chemokine receptor CCR7 and α4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes

Kathleen J. Till, Ke Lin, Mirko Zuzel, and John C. Cawley

Malignant lymphocyte migration into lymph nodes is an important aspect of chronic lymphocytic leukemia (CLL), yet little is known about the processes involved. Here we demonstrate that CLL cells migrate across vascular endothelium in response to at least 3 chemokines, namely, CCL21, CCL19, and CXCL12. Moreover, transendothelial cell migration (TEM) in response to CCL21 and CCL19 was significantly higher for the malignant B cells of patients who had clinical lymph node involvement as compared with those of patients lacking such organomegaly. Furthermore, the expression of CCR7, the receptor for both CCL21 and CCL19, correlated with clinical lymphadenopathy, and blocking of CCR7 inhibited CLL cell TEM. By using immunohistochemistry we demonstrated that CCL21 and CCL19, but not CXCL12, are located in high endothelial venules and are, therefore, in an appropriate location to induce TEM. Regarding the adhesion receptors involved in TEM, α4 (most likely in association with β1) and αLβ2 were shown to be important in CLL cell TEM in vitro, but only the level of α4 expression correlated with the presence of clinical lymphadenopathy. The present studies are the first to shed light on the factors determining CLL cell entry into nodes and define the phenotype of circulating malignant cells likely to determine the pattern of lymph node enlargement in the disease.

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From the Department of Haematology, University of Liverpool, Liverpool, United Kingdom.


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Reprints: Kathleen J. Till, Department of Haematology, Royal Liverpool University Hospital, Daulby Street, Liverpool, L69 3GA, United Kingdom; e-mail: kjtill@liv.ac.uk.

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CXCL10 (also known as monokine induced by γ interferon) and CXCL10 (also known as interferon-inducible protein 10), these factors are inflammatory chemokines and are not associated with HEV. CCR6 and its ligand CCL20 (MIP-α; also known as MIP-3α) have been implicated in B-cell migration. However, expression of this chemokine is associated with inflamed epithelium and inflammatory cells and was, therefore, thought unlikely to be involved in CLL cell TEM. Here we present data suggesting that the CCR7 ligands CCL21 and CCL19 are likely to be important for CLL cell transmigration across HEVs into nodes. Furthermore, the expression levels of CCR7, and also those of the α4-integrin chain on CLL cells, were significantly higher in patients with clinical lymphadenopathy compared with those without lymph node disease. This study, therefore, identifies a chemokine receptor and an integrin chain associated with peripheral lymph node enlargement in CLL.

### Patients, materials, and methods

**Patients**

Peripheral blood from 30 cases of CLL was studied with informed consent. The diagnosis was based on the presence in the blood of morphologically typical lymphocytes expressing low-density, light-chain restricted, surface immunoglobulin, together with CD5 and CD23.

Clinical details, including the presence or absence of lymph node enlargement (>1 cm at 2 or more sites as detected by clinical examination) and \( V_\delta \) gene status, are listed in Table 1. This table also lists the relevant phenotypic data obtained in the present study to allow assessment of their relationships to clinical disease.

### Cell preparation and culture

**CLL cells.** CLL cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation and stored in liquid nitrogen before use. To ensure high CLL cell purity, only patients with counts of more than 50 × 10⁹/L were studied. Frozen cells were rapidly thawed at 37°C and slowly reconstituted in RPMI containing 1% bovine serum albumin (BSA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Paisley, United Kingdom). Cells were then allowed to recover for 60 minutes at 37°C in 5% CO₂ in air (recovery times of 30, 60, and 120 minutes were tested and transmigration was similar at 60 and 120 minutes and greater than at 30 minutes). In selected patients (n = 3), both fresh and frozen cells were studied. Very similar transmigration was observed (data not shown).

**Human umbilical vein endothelial cells.** Endothelial cells were stripped from the vein with trypsin and were cultured to confluence in Iscoves modified minimal essential medium containing 20% newborn calf serum, 2 mM L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 15 μg/mL endothelial cell growth factor (Life Technologies). Human umbilical vein endothelial cells (HUVECs) that had been passaged up to 3 times were used in the transmigration assays.

### Table 1. Clinical details of patients with chronic lymphocytic leukemia

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<th>Nodes</th>
<th>Treatment</th>
<th>Age (y)</th>
<th>Hb (g/dL)</th>
<th>WBC (× 10⁹/L)</th>
<th>CD38 (%)</th>
<th>( V_\delta ) mutation*</th>
<th>( \alpha 4/\beta 7 ) (MFI)</th>
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Hb indicates hemoglobin; WBC, white blood cell count; MFI, mean fluorescent intensity; MI, migration index; CLB, chlorambucil; CHOP, cyclophosphamide, hydroxydaunorubicin, Oncovin, and prednisone; FMD, fludarabine, mitoxantrone, and dexamethasone; NA, not available; IFN, interferon; CVP, cyclophosphamide, vincristine, and prednisone.

*\( V_\delta \) unmutated cases presents the percentage of deviation from the most similar germline \( IgV_\delta \) gene in the V BASE directory.†

†Stage at study. No organomegaly and stable, therefore, was regarded as Binet stage A.

‡Staged as C/IV on the basis of persistent thrombocytopenia.
Chemokines and antibodies

The following chemokines were used: CCL21, CCL19, CXCL12, and CXCL8 (interleukin-8) (all from R&D Systems, Oxford, United Kingdom). Anti-CCR7 (goat polyclonal), CCL19, and CXCL12 (monoclonal antibody [mAb]; immunoglobulin G [IgG]2b and IgG1, respectively), and vascular cell adhesion molecule 1 (VCAM-1; goat polyvalent) (all from R&D Systems) were used for tissue staining. The following biotinylated second-layer antibodies were used: rabbit antigoat (Vector Laboratories, Peterborough, United Kingdom) and goat antimouse (Zymed, Cambridge, United Kingdom). Nonspecific goat immunoglobulin and mouse IgG1 and IgG2b were used as controls (all from R&D Systems).

A mAb (IgM; Pharmingen, Oxford, United Kingdom) was used to examine CLL cell expression of CCR7, the receptor for both CCL21 and CCL19; antibody staining was detected with fluorescein isothiocyanate (FITC)-conjugated goat antimouse immunoglobulin. An FITC-conjugated anti-CD19 (IgG1; Becton Dickinson, Oxford, United Kingdom) was used to determine both CLL cell purity and the nature of cells migrating in the TEM system (because only very high count cases were used, proportionally very few normal B cells were present in the cell preparations). For both mAbs, fluorescence was measured by fluorescence-activated cell sorter (FACS), with inclusion of nonspecific IgM and IgG1 (Becton Dickinson) as class-specific controls. Expression of αL (IgG2a), α4 (IgG2b), and β7 (IgG2a) integrin chains and of L-selectin (IgG2a) was measured with specific mAbs (all mAbs and isotypic controls from Becton Dickinson) and FACS analysis. Because we have previously shown that the level of expression of these integrins by CLL cells is low,2 a 3-layer technique using biotinylated horse antimouse antibody (Vector Labs) as second layer and streptavidin-phycocerythrin (Becton Dickinson) as the final layer were used. Nonspecific IgG2a and IgG2b (Becton Dickinson) were used as controls.

In addition, mAbs were used to determine which integrins and chemokine receptors might be involved in TEM. Blocking mAbs against the following were used: αL (IgG2a), α4 (IgG1) (both from R&D Systems), α5 (IgG1; Pharmingen), β1 (IgG2b), β2 (IgG1) (both from R&D Systems), β7 (IgG1; Pharmingen), and CCR7 (rat IgG2a) (3D12, a kind gift from M. Lipp, MD, Berlin). Nonspecific mouse IgG1, IgG2a, and IgG2b were used as controls (all from R&D Systems).

TEM assay

HUVECs were grown to confluence on the inserts of Transwell plates (5-μm pore size; Corning Costar, High Wycombe, United Kingdom). The HUVECs were washed in RPMI, and 5 × 10⁴ CLL cells (in RPMI containing 0.1% BSA, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) were added to the inserts. Chemokines were then added to the bottom wells at the following concentrations: CCL21 and CCL19 at 10, 100, 1000, and 2000 ng/mL; CXCL12 at 10, 100, and 1000 ng/mL; and CXCL8 at 0.5, 5, and 50 ng/mL (all in RPMI containing 0.1% BSA, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin). It was shown in preliminary experiments that maximal migration indices (MIs) were observed at CCL21 and CCL19 concentrations of 1000 ng/mL and at a CXCL12 concentration of 100 ng/mL. These concentrations of chemokine were, therefore, used in all subsequent experiments. No TEM was observed at any of the concentrations of CXCL8 (previous work from this department has shown that CXCL8 at 5 ng/mL induces maximal CLL cell movement on the extracellular matrix component hyaluronan). A range of incubation times was also tested (2, 4, 6, 8, and 24 hours); the MI was maximal at 6 hours and remained constant against longer incubation times; therefore, an incubation time of 6 hours was routinely used. All assays were performed in triplicate.

After incubation, the underside of the inserts were scraped to remove any cells that had recently transmigrated, and the cells were then harvested from the bottom wells. As some CLL cells had adhered to the bottom wells, EDTA (0.2%; Sigma, Poole, United Kingdom) was added for 5 minutes at 37°C before harvesting. These transmigrated cells were then counted in a hemocytometer. To determine the CLL cell content of the transmigrated cells, an aliquot was always stained for CD19 and analyzed by FACS. The MI (no. of CD19⁺ cells transmigrating with chemokine divided by no. of cells transmigrating in the absence of chemokine) was then calculated. Student t test was used to determine the statistical significance of the results.

Inhibition of TEM

CLL cells were incubated at 4°C for 30 minutes with blocking mAbs (concentrations) to the following integrin chains: β1 (1 and 10 μg/mL), β2 (1 and 10 μg/mL), β7 (1 and 10 μg/mL), α4 (0.5 and 5 μg/mL), α5 (0.2 and 2 μg/mL), and αL (1 and 10 μg/mL). Also, in certain experiments a blocking mAb to CCR7 was used (50 and 100 μg/mL). These amounts of mAbs were chosen to cover the range of concentrations that have been reported to block adhesion in other systems (manufacturers’ recommendations and M. Lipp, written personal communication, October 2001). CLL cells were then added to the Transwell inserts, and the TEM assay was performed as above.

In addition, CLL cells were incubated for 2 hours at 37°C with 1 μg/mL pertussis toxin (Sigma) to block chemokine receptor signaling and were washed. TEM measurements were performed as above.

Tissue staining

CLL nodes (n = 5) were diagnostic samples. Normal nodes (n = 5) were obtained from axillary clearance for breast cancer and were macroscopically and microscopically normal.

Formalin-fixed paraffin-embedded tissue was stained for chemokines as follows. After clearing and rehydration, slides were boiled in 10 mM sodium citrate buffer (pH 6) for 10 minutes and blocked with 10 mg/mL BSA before overnight incubation with antibodies to CCL21 (20 μg/mL) and VCAM-1 (1:200) at 4°C, or with mAbs to CCL19 (5 μg/mL) and CXCL12 (50 μg/mL) at 25°C. These amounts of the antibodies were chosen after titration over a range of concentrations. Sections were then incubated with biotinylated rabbit antigen (CCL21 and VCAM-1) or with biotinylated goat antimouse (CCL19 and CXCL12) antibodies, followed by ExtraAvidin alkaline phosphatase (Sigma) and exposure to substrate (Fast Red/Naphthol AS MX phosphate/levamisole; Sigma). Slides were counterstained with hematoxylin (Sigma).

V₄H hypermutation analysis

For each case, total RNA was extracted from CLL cells by using Trizol reagent (Life Technologies) and 1-μg aliquots were reverse transcribed with M-MuLV reverse transcriptase (Promega, Southampton, United Kingdom) and an oligo(dT)₁₅ primer. Aliquots of the resulting complimentary DNAs were used to isolate clonally expressed VH DH JH sequences in 2 sets of reactions. The first set of 7 reactions, the sense primers were consensus sequences obtained from axillary clearance for breast cancer and were macroscopically and microscopically normal.

V₄H gene hypermutation analysis

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Results

CCL21, CCL19, and CXCL12 all induce CLL cell TEM

In the absence of chemokine, CLL cells showed little TEM (< 0.2%) in the Transwell migration assay. Variable CLL cell TEM (0%-12% of input cells) was seen in response to CCL21, CCL19, and CXCL12 (n = 8 cases; Figure 1). When CLL cell migration was observed, CCL21 and CCL19 produced comparable MIs, which were usually higher than those induced by CXCL12. As expected from our previous work showing that CXC8 stimulates CLL cell movement within, but not into nodes, this chemokine failed to induce CLL cell TEM in any of the cases studied (Figure 1).

Because the TEM induced by CCR7 stimulation seemed of potential in vivo relevance (more to follow), the reproducibility of cell migration in response to CCL21 was studied on 2 or more occasions in all 8 cases. When the MI was low (< 10; n = 4), it remained low on repeated testing. Similarly, when the MI was high (> 20; n = 4), it remained high but varied at different times of study (eg, in case 7 studied 5 times, the MI was between 48 and 120). CCL21-induced CLL cell TEM was completely abrogated by preincubation of CLL cells with pertussis toxin (MI = 0.7 ± 0.3; n = 3), an inhibitor of chemokine-receptor signaling.

Clinical analysis of the patients studied in Figure 1 indicated that the 4 patients whose cells repeatedly migrated in response to CCL21 and CCL19 all had lymph node enlargement, whereas those cases whose cells displayed little or no transmigration had no lymphadenopathy. Because at the onset of the present study CCL21, known to be concentrated in HEV, seemed to be the more likely candidate chemokine to induce CLL cell TEM in vivo, we examined the relationship between migration to CCL21 and clinical lymphadenopathy in a larger number of patients.

CCL21-mediated TEM of CLL cells is related to clinical lymphadenopathy

When an additional 22 CLL patients were studied, the mean MI was significantly higher in patients with nodal enlargement compared with those without lymphadenopathy (31 versus 10; P = .001; Figure 2A). This finding demonstrates that the ability of CLL cells to cross endothelium is related to the presence of clinical lymphadenopathy. As expected, because lymphadenopathy is one of the features used in clinical staging, there was also an association between high MI and later stages of the disease (stage 0 versus I-IV = 8.4 ± 0.2 versus 27 ± 6, P = .002).

In view of the recent interest in the fact that CLL can be divided into 2 very different prognostic groups according to the extent of V_H gene hypermutation, we next examined the relationship between such hypermutation and TEM. In the patients (n = 26) for whom V_H data were available, there was no significant difference (P > .05; Figure 2B) between CLL cell TEM in patients with or without hypermutation regardless of whether a threshold of 2% or 5% deviation from the germline was used in the analysis. However, more CLL patients with clinical lymphadenopathy had nonhypermutated V_H genes (10 of 15 at < 2% V_H mutation and 13 of 15 at < 5% V_H mutation) than did those without nodes (3 of 11 at < 2% V_H mutation and 4 of 11 at < 5% V_H mutation). When the proportions were analyzed by Fisher exact test, the association between lymphadenopathy and V_H nonhypermutation was significant when the 5% mutation threshold was used (P = .01) but not at the 2% threshold level (P = .11). We, therefore, concluded that the ability of CLL cells to migrate into, or accumulate in, nodes may be related to their V_H hypermutational status.

Because expression of CD38 is a poor prognostic indicator in CLL and is often associated with the absence of V_H hypermutation, we also examined the relationship between CD38 expression, TEM, and nodal disease. We found that, although there was a trend toward an association between high CD38 expression and lymphadenopathy (mean ± SE for patients with lymphadenopathy = 20.8 ± 6.3% CD38+ cells; for those without enlarged lymph nodes 6.8% ± 4.6%), it did not reach statistical significance (P = .08). There was no association between high CD38 expression (> 30% positive cells) and TEM (Figure 2C; n = 27; P = .29), reflecting that not all patients with lymphadenopathy displayed high CD38 expression, and yet in all cases the malignant cells migrated in response to CCL21.

In conclusion, the data show that clinical lymphadenopathy is clearly related to the ability of CCL21-stimulated CLL cells to undergo TEM. In addition, it seems that less mature non-V_H hypermutated and CD38+ CLL cells have a greater propensity to accumulate in nodes than do more mature V_H hypermutated and CD38- cells.

We next investigated the mechanism(s) responsible for the different CLL cell migratory responses to CCL21 observed in patients with lymphadenopathy, compared with those lacking lymph node enlargement. It has been shown in adult T-cell leukemia that CCL21 receptor (CCR7) expression is related both to the malignant cell chemotactic response to CCL21 and to organ involvement in the disease. We, therefore, examined the CCR7 expression of the CLL cells in our series of patients and related receptor levels to the presence or absence of lymphadenopathy.
CCR7 expression is higher in patients with lymphadenopathy

Variable CCR7 expression was detected on the CLL cells from all of the patients examined. In most cases, a distinct positive peak consisting of more than 95% of cells was observed. In 5 cases in which the cells had a mean fluorescence intensity (MFI) less than 20, a peak shift was seen, indicating that virtually all (>95%) of the cells were weakly positive.

The intensity of CCR7 expression was significantly higher on the CLL cells of patients with lymphadenopathy compared with those without lymph node enlargement (Figure 3 and Table 1). This finding suggests that transmigration to lymph nodes involves stimulation of CCR7. We, therefore, used an anti-CCR7 blocking mAb in TEM experiments and showed that this reagent strongly reduced CLL cell migration to CCL21 (Figure 3B).

Because both CCL21 and CCL19 are ligands of CCR7, we next examined the expression of these 2 chemokines in CLL and normal nodes. In addition, CXCL12 also stimulated CLL cell TEM, we examined the distribution of this CXCR4-binding chemokine.

CCL21 and CCL19, but not CXCL12, are associated with HEVs

In CLL nodes, the normal architecture is completely replaced by infiltrating leukemic cells among which a variety of vascular and lymphatic channels are visible. In both CLL and normal nodes (Figure 4), weak staining for both CCL21 and CCL19 was found in HEVs; in contrast, these vessels were negative for CXCL12. The stroma was reactive for all these chemokines with some stromal cells, including the peri-HEV fibroblasts, showing strong staining for CCL21 (Figure 4).

We, therefore, concluded that the location of CCL21 and CCL19, but not CXCL12, indicated that these chemokines can mediate CLL cell entry into lymph nodes. Thus, these histologic findings add relevance to our in vitro observations that migration to CCL21 and CCL19, and expression of the receptor (CCR7) for these chemokines, is related to the presence of lymphadenopathy in CLL. However, although the levels of CCR7 were significantly different between the patients with and without lymphadenopathy, there was some overlap in expression between the 2 groups. We, therefore, examined other factors that might be involved in determining the entry of CLL cells into lymph nodes in vivo.

We first examined the expression of L-selectin by CLL cells because this molecule is essential for the initial tethering of lymphocytes to HEVs before integrin-mediated arrest and subsequent diapedesis across endothelium.

L-selectin expression by CLL cells is not correlated with clinical lymphadenopathy

FACS analysis showed that L-selectin expression by CLL cells varies greatly from case to case. In all patients studied, at least a proportion (16%-82%) of cells expressed variable levels of L-selectin (MFI, 30-176). However, there was no correlation between the percentage positivity ($P = .3$), or MFI ($P = .5$), and the presence or absence of lymphadenopathy (data not shown).

As regards integrin involvement in lymphocyte TEM, the most important receptors are $\alpha_4\beta_1$ and $\alpha_4\beta_2$. We and others have previously shown that CLL cells consistently express $\alpha_4\beta_2$, whereas expression of $\alpha_4$, in combination with either $\beta_1$ or $\beta_7$ chains, is variable. We, therefore, examined whether these integrins, as in normal lymphocytes, were involved in CLL cell TEM.
**Discussion**

The present study is concerned with the process by which CLL cells enter lymph nodes. We demonstrated that a number of relevant chemokines (CCL21, CCL19, and CXCL12) can induce the malignant cells of a proportion of cases of CLL to migrate through vascular endothelium. However, we found that CCL21 and CCL19 induce more CLL cells to transmigrate than does CXCL12. Furthermore, we found that the ability of CLL cells to respond to CCL21 and CCL19 in vitro strongly correlates with the presence of clinical lymphadenopathy. We then went on to show that, although all CLL cells possess the receptor for these chemokines (CCR7), levels of expression are higher on the cells of patients with lymph node enlargement. These results are entirely compatible with a study in adult T-cell leukemia43 which found that the ability of the malignant cells to respond to CCL21 correlated with CCR7 expression and lymph node enlargement. Moreover, by using a blocking antibody, we now directly demonstrate involvement of this receptor in CLL cell TEM in response to CCL21.

In view of the current interest in the pathogenetic and prognostic significance of $\mathrm{V_H}$ hypermutational status and CD38 expression in CLL,40,41,47,48 and because lymph node enlargement is a feature of more advanced disease and poor prognosis, we also examined whether these parameters are related to the degree of in vitro TEM and clinical lymphadenopathy. We found that there was no correlation between $\mathrm{V_H}$ hypermutation and TEM, but more patients with lymphadenopathy had nonmutated $\mathrm{V_H}$ genes than did patients without nodes. These findings are compatible with previous reports that lymphadenopathy can be found in patients with or without $\mathrm{V_H}$ hypermutation,40,46 As for CD38 expression, the cells of patients with enlarged lymph nodes expressed more CD38 and had higher levels of TEM than did patients without lymphadenopathy. However, only the relationship between $\mathrm{V_H}$ nonhypermutation (< 5%) and lymphadenopathy achieved statistical significance. With regard
to CD38 expression and lymphadenopathy, it is interesting that a recent report of a very large number of CLL cases showed a significant positive correlation between these 2 parameters.42

By using immunohistochemistry, we next investigated the location of CCL21, CCL19, and CXCL12 in CLL and normal lymph node tissue. CCL21 and CCL19 were found in both HEVs and the surrounding stroma, whereas CXCL12 was found in stroma only. These findings, together with our demonstration of the correlation between CCR7 expression and lymphadenopathy, suggest that CCL21 and/or CCL19 are likely to be involved in the stimulation of CLL cell entry into lymph nodes. These conclusions are in accord with studies in knockout mice, suggesting that CCR7 is important for the migration of B cells into nodes.21 Furthermore, our immunohistochemical observations are compatible with previous studies in mice showing that, of the 2 chemokines, CCL21 is produced in HEV and that CCL19 produced by stromal cells is transcytosed through endothelium to the luminal surface of HEVs.49 Although our data suggest that CCR7 ligand(s) are important in CLL cell TEM, they do not rule out the possibility that other chemokines such as CXCL13 (for which CLL cells express approximately 40% of patients, a finding compatible with the approximate 50% of our cases expressed α4), but they have not attempted to relate α4 expression to lymphadenopathy. We, therefore, conclude that α4 expression (most likely in association with β1), along with CCR7 receptor levels, play an important role in CLL cell entry into lymph nodes and in the generation of clinical lymphadenopathy.

There have been a number of previous studies of integrin expression on CLL cells.5,45,53 Most demonstrate α4 on the cells of approximately 40% of patients, a finding compatible with the present study (approximately 50% of our cases expressed α4), but they have not attempted to relate α4 expression to lymphadenopathy. We believe, therefore, that the present study is the first to demonstrate an association in CLL between malignant cell expression of a particular integrin chain and clinical lymph node enlargement.

In conclusion, then, the present study suggests that CCR7 engagement by CCL21 and/or CCL19 stimulates CLL cell entry into lymph nodes by HEV and that CCR7 and α4 expression are important for this process. Although we believe that the present findings are important for the entry of CLL cells into nodes, further studies are needed to determine why the malignant cells accumulate in, rather than exit from, lymph nodes in patients with clinically detectable lymphadenopathy.

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References


The chemokine receptor CCR7 and α4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes

Kathleen J. Till, Ke Lin, Mirko Zuzel and John C. Cawley